P#33

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From: Kate . Murashige

Date: July 5, 2002
Time: 11:13 AM

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C mments: 51158-20031.00

PLEASE DELIVER TO EXAMINER NICKOL FOR 7/8/02 MEETING.

Re:**09/410,132, 09/323,597 and 09/615,285**

We look forward to meeting with you Monday, July 8th at 11:15a.m.

Enclosed is a declaration for your review to address the outstanding issue in 09/410,132 (Protein 22P4F11).

09/323,597 and 09/615,285 / TMPR/SS2---we just want to confirm we are on course (no outstanding office actions).

Docket No. 511582803100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**DECLARATION OF PIA M. CHALLITA-EID
UNDER 37 C.F.R. § 1.132:
REGARDING INTERPRETATION OF NORTHERN BLOT DATA**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Pia M. Challita-Eid, declare as follows:

1. I have a Ph.D. in Microbiology from University of Southern California, did post doctoral work at University of California at Los Angeles, and was a faculty member at the University of Rochester. I have been practicing in the field of molecular biology for over 10 years. At Agensys, I am the Group Leader of Gene Discovery. In my position at Agensys, I have responsibility for evaluating the levels of expression of various genes in tissues. A copy of my *curriculum vitae* is enclosed as Exhibit A.

2. Our company, Agensys, is dedicated to discovery of proteins that are highly expressed in various tumor tissues as compared to normal tissues. The company approaches this discovery task by first identifying cDNAs which correspond to genes overexpressed in tumor tissue using the technique of suppression subtractive hybridization (SSH). In this technique, cDNA from normal tissues is subtracted from cDNA from tumor tissues. Thereby, cDNA present in tumor tissues, but not in normal tissues is isolated. Thus, on a gene-by-gene basis, this approach can indicate that a gene corresponding to the cDNA is overexpressed in tumor cells.

3. Typically, the next step is to utilize the sequence information obtained from SSH to obtain a full-length DNA clone which includes the entire open reading frame for the protein corresponding to this cDNA.

4. In addition, the level of expression of the corresponding gene is determined in various normal tissues and in various tumor tissues and tumor cell lines using the technique of Northern blotting, which detects production of messenger RNA. It is well known that the production of messenger RNA, that encodes the protein, is a necessary step in the production of the protein itself. Therefore, detection of high levels of messenger RNA by, for example, Northern blot, is a way of determining that the protein itself is produced.

5. Northern blotting is a detection method of relative levels of mRNA expression of a gene. It is procedure in which specific mRNA is measured using a nucleic acid hybridization technique. The signal is detected on an autoradiogram. The stronger the signal, the more abundant is the mRNA. For genes that produce mRNA that contains an open reading frame flanked by a good Kozak translation initiation site and a stop codon, in the majority of cases the synthesized mRNA is expressed as a protein. Kozak translation initiation sites are discussed in greater detail paragraph 7, below.

6. The evidence referred to in paragraphs 3, 4 and 5 above is consistent with the general knowledge in the art of molecular biology that, with rare exceptions, expression of an mRNA is predictive of expression of its encoded protein. This is particularly true for mRNA with an open reading frame and a Kozak consensus sequence for translation initiation.

7. The consensus Kozak initiation site CCACC*ATG*G where the ATG start codon is italicized, refers to the "optimum" translation initiation sequence. A study by Peri and Pandey *Trends in Genetics* (2001) 17: 685-687, describes a study of over 1500 translation initiation sites in order to address the natural mRNA translation initiation. This study showed that the most authentic initiation sequence has 3 or less mismatches from the

optimum consensus Kozak sequence CCACCATGG. The sequence of the translation initiation site of 22F4F11, TCAGC***ATG***G, shows only 2 nucleic acid differences from the optimum Kozak consensus. Also, the translation initiation site of 22P4F11 contains a G at position +4, which has been shown to significantly augment translation efficiency (Kozak (1997) Embo J 16:2482-92). Altogether, these data demonstrate that the translation initiation site of 22P4F11 is functional and can initiate protein translation.

8. The Northern blot technique is used as a routine procedure (as compared to Western blotting, immunoblotting or immunohistochemistry) because it does not require the time delays involved in isolating or synthesizing the protein, preparing an immunological composition of the protein, eliciting a humoral immune response, harvesting the antibodies, and verifying the specificity thereof. All of these things can be done, but they take time, and the presence of mRNA on Northern blots, especially in comparative tissues, is a recognized indication that the protein itself will be produced.

9. I am familiar with the general practice of Northern blotting and interpretation, described above, being carried out, not only at Agensys, but also at other companies that seek to evaluate gene expression in various tumor and other tissues. The use of Northern blots as a means for evaluating protein production is universally accepted as reliable and is therefore widely practiced.

10. It is understood that the absolute levels of messenger RNA present and the amounts of protein produced do not always provide a 1:1 correlation. However, in those instances where the Northern blot has shown mRNA to be present, it is almost always possible, in my experience, when the time is taken to do so, to detect the presence of the corresponding protein in the tissue which provided a positive result in the Northern blot. The levels of the protein compared to the levels of the mRNA may be disjunctive, but is inaccurate to say that there is no correlation between protein levels and mRNA levels as a general matter. In general, cells that exhibit detectable mRNA also exhibit detectable

corresponding protein and *vice versa*. This is particularly true where the mRNA has an open reading frame and a good Kozak sequence.

11. Ironically, studies seeking to determine the overall pattern of correlation between mRNA and corresponding protein have generally started with displaying the protein fingerprint of a particular cell or tissue. For instance, an article by Anderson, L. and Seilhamer, J., *Electrophoresis* (1997) 18:533-537 (Exhibit B) describes such a study on a patient liver. A 2D gel was obtained to determine the pattern of proteins in the liver, and a cDNA library was used to determine the pattern for mRNA. The authors found that of 23 selected proteins which could be identified from the gel, mRNA for 19 were detected in the transcript images. Thus, in the vast majority of cases, there was both mRNA and protein present. The authors found that the levels of RNA units to protein units had a correlation coefficient of 0.48. As they state, this number is intriguingly close to the middle position between a perfect correlation (1.0) and no correlation whatever (0.0). Only a correlation coefficient of (0.0) would support a proposition that mRNA presence provides no indication of protein presence. The conclusion has to be that in the vast majority of instances, i.e., any correlation coefficient other than (0.0), where mRNA is present protein is also present. It is inaccurate to say that there is no correlation between mRNA expression and protein expression.

12. An article by Oh, J.M.C., *et al., Proteomics* (2001) 1:1303-1319 reports a database of protein expression in lung cancer. Again, the study sought to determine the correlation between mRNA and corresponding protein beginning with protein fingerprint display of a particular cell or tissue. Protein expression was evaluated using 2D gels and mRNA expression was evaluated using microarrays. The approach is suggested as a general tool for evaluating the correlation between mRNA expression and protein expression. Clearly the fundamental premise of the approach is that the correlation will not be zero, or the tool would not even be proposed, let alone published in a peer-reviewed journal.

13. I am aware of publications such as Fu, L., *et al., Embo. Journal* (1996) 15:4392 - 4401 which reports an extremely rare occurrence where there appears to be zero production of any protein even in the presence of mRNA. This is for the specific protein p53. I am not familiar with any other instances where this occurs. This is an exception to the rule that there is at least some correlation between mRNA presence and protein production. This is supported by the publication itself; were this not an unusual occurrence, this lack of correlation would not have merited publication at all.

14. In many cases, a reported lack of protein expression is due to technical limitations of the protein detection assay. For instance, the available antibody may only detect denatured protein but not native protein present in a cell. In other instances, the half-life of the protein is very short; thereby the steady-state protein levels are below detectable range. Short-lived proteins are still functional, and some have been previously described to induce tumor formation as shown in the article by Reinstein, *et al., Oncogene 19: 5944-50.* In such situations, when more sensitive detection techniques are performed and/or other antibodies are generated, protein expression is detected. When studies fail to take these principles into account, they are likely to report artifactually lowered correlations of mRNA to protein.

15. Most genes, when they produce mRNA that contains an open-reading frame flanked by a good Kozak translation initiation site and a stop codon, the synthesized mRNA code for a protein. Analysis of 22P4F11 shows a strong mRNA signal on Northern blot in cancer tissues, and the mRNA sequence shows an open-reading frame containing a good Kozak initiation site and a stop codon. Therefore, production of 22P4F11 protein is reasonably predicted based on this data.

16. In summary, the scientific community regards the presence of mRNA in cells is indicative of the production of protein. This is particularly true when the Northern data are strong and the mRNA has an open reading frame and a good Kozak sequence. It is understood that the correlation of mRNA and protein levels is not perfect, however, an instance such

as that in Fu, where protein appears to be absent although mRNA is present, is a rare exception.

17. The use of positive Northern blots as both indicative of and predictive of protein production is a recognized conclusion of scientists in this field.

18. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Santa Monica, California on 26 June 2002.

Pia M. Challita-Eid

51158-28031.00

Agensys, Inc.

MEMORANDUM

To: Kate Murashige
From: Tim Lithgow
Date: June 26, 2002
Re: 51158-20031.00

Here is the Executed Declaration for filing on 22P4F11.